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Byssochlamys nivea with patulin-producing capability has an isoepoxydon dehydrogenase gene (*idh*) with sequence homology to *Penicillium expansum* and *P. griseofulvum*

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ABSTRACT

Nucleotide sequences of the isoepoxydon dehydrogenase gene (*idh*) for eight strains of *Byssochlamys nivea* were determined by constructing GenomeWalker libraries. A striking finding was that all eight strains of *B. nivea* examined had identical nucleotide sequences, including those of the two introns present. The length of intron 2 was nearly three times the size of introns in strains of *Penicillium expansum* and *P. griseofulvum*, but intron 1 was comparable in size to the number of nucleotides present in introns 1 and 2 of *P. expansum* and *P. griseofulvum*. A high degree of amino acid homology (88 %) existed for the *idh* genes of the strains of *B. nivea* when compared with sequences of *P. expansum* and *P. griseofulvum*. There were many nucleotide differences present, but they did not affect the amino acid sequence because they were present in the third position. The identity of the *B. nivea* isolates was confirmed by sequencing the ITS/partial LSU (28 S) rDNA genes. Four *B. nivea* strains were analysed for production of patulin, a mycotoxin found primarily in apple juice and other fruit products. The *B. nivea* strains produced patulin in amounts comparable to *P. expansum* strains. Interest in the genus *Byssochlamys* is related to the ability of its ascospores to survive pasteurization and cause spoilage of heat-processed fruit products worldwide.

Introduction

The genus *Byssochlamys* contains two economically important species, *B. nivea* and *B. fulva*. Both species cause spoilage of processed fruit products and are among the most commonly encountered fungi associated with spoilage of heat-processed fruits in countries worldwide (Tournas 1994). The ascospores of *B. fulva* are capable of resisting a temperature of 87 °C for 30 min in fruit juices, but conidia are killed at this temperature (Olliver & Rendle 1934). Because the ascospores can survive the heat treatments that are used for fruit processing, they can germinate and grow, spoiling products at room temperature during storage. Pressures above 600 MPa and temperatures above 60 °C were needed for inactivation of *B. nivea* ascospores (Butz *et al.* 1996). *B. nivea* and *B. fulva* are present in soil in fields and orchards, the initial source of contamination. Their growth proceeds rapidly in the processed fruit where conditions (acidity and sugar) are ideal. *B. fulva* can also grow under reduced oxygen tension (King *et al.* 1969). Although cold storage at 0–2 °C for several months has been found to inhibit growth, the fungus is not killed (Olliver & 2Rendle 1934).

Patulin, a toxic secondary metabolite, can be produced by *B. nivea* and *B. fulva*, as well as several species of *Penicillium*

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and *Aspergillus* (Jackson & Dombrink-Kurtzman 2006). *Byssochlamys nivea* has been reported to be more likely to produce patulin than *B. fulva* (Rice *et al.* 1977). Apple juice serves as an excellent substrate for growth of *B. nivea*, followed by production of the mycotoxin patulin over a temperature range of 12–37 °C (Roland & Beuchat 1984a,b). If these fungi contaminate fruit juice before thermal processing, ascospores can survive the processing, and the surviving cells may produce patulin in the finished product. (Harrison 1989). The conjugation of patulin to sulphydryl and primary amino groups has been reported to cause chromosomal aberrations (Fliege & Metzler 2000). There have been reports that patulin can have mutagenic, carcinogenic and teratogenic properties, but this is still a matter of debate (Pfeiffer *et al.* 1998). Long-term testing of patulin administered directly to the forestomach of Sprague-Dawley rats was able to produce a low level of benign tumours, but no carcinogenic action was observed (Becci *et al.* 1981). Interestingly, when pregnant Swiss mice received a total dosage of 24 mg patulin kg^{-1}, their offspring showed no evidence of carcinogenic effects, but during the neonatal period (within 2–6 days of birth), 11 of the 52 females and eight of the 43 live birth males died, all showing similar signs of toxicity (Osswald *et al.* 1978).

Interest in fungi able to produce patulin is due to occurrence of patulin in stored apple juice. In the United States, the present regulatory level for patulin in apple juice and apple products is 50 $\mu g\,kg^{-1}$. When patulin is present in commercial products at a higher level, the US Food and Drug Administration (FDA) has the authority to remove the products from commerce. In the European Union (EU), the regulatory level for patulin in apple juice and apple products has recently been lowered to 25 $\mu g\,kg^{-1}$; a lower level of 10 $\mu g\,kg^{-1}$ has been established for infants and small children (Arranz *et al.* 2005).

The purpose of this research was to determine the nucleotide sequence of the isoepoxydon dehydrogenase (*idh*) gene of the patulin biosynthetic pathway for comparison with the sequence of the *idh* gene in *P. expansum* and *P. griseofulvum*. Isoepoxydon dehydrogenase is the seventh of ten enzymes in the direct pathway involved in the synthesis of patulin. Isoepoxydon dehydrogenase requires the co-factor $NADP^+$ for the conversion of isoepoxydon to phyllostine (Fedeshko 1992; White *et al.* 2006). Eight different strains of *B. nivea* were examined, and the identity of each was verified from nucleotide sequences of the ITS regions 1 and 2 and partial LSU (28 S) rDNA.

Materials and methods

Fungal strains

The nucleotide sequence of the *idh* gene was determined for eight different strains of *Byssochlamys nivea*. Included were NRRL 32565^T (ex-type strain), NRRL 2615, NRRL 29820, NRRL 32294, NRRL 35216, NRRL 35233, NRRL 35592 and NRRL 35593. Isolates used in the study are maintained in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, and are listed in Table 1.

Table 1 – List of strains and their sources

Species names	Strain designations			Sources
	NRRL	CBS	ATCC/UCD	
Byssochlamys nivea	32565^T	100.11^T	22260^T	Unknown
B. nivea	2615		56268	Unknown
B. nivea	29820			Unknown
B. nivea	32294		36614	Silage
B. nivea	35216	546.75		Unknown
B. nivea	35233		42746	Unknown
B. nivea	35592		40–200[a]	Unknown
B. nivea	35593		55–85[a]	Canned fruit

NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL. ATCC, American Type Culture Collection, Manassas, VA. CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. ^{T}, ex-type strain.
a UCD, Herman Phaff Culture Collection, University of California, Davis, CA.

Isolation of genomic DNA

For determination of the *idh* gene sequences of eight isolates of *Byssochlamys nivea*, total fungal DNA was isolated following growth on potato dextrose agar (PDA) plates for approximately two weeks at 30 °C. To each plate was added, 2–3 ml of 70 % ethanol. Surface growth was brought into suspension using a sterile rod, bent at a 90° angle. The suspension was put into a 1.5 ml Eppendorf tube and centrifuged for 5 min to precipitate the cellular material. The supernatant was discarded and 375 µl DNA extraction buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA, pH 8.5, 0.5 % SDS) and 125 µl glass beads (diam 0.5 mm; Scientific Industries, Bohemia, NY) were added to the pellet. Tubes were vortexed on a TurboMix (Scientific Industries) for 5–10 min and 350 µl 2× CTAB (hexadecyltrimethylammonium bromide) buffer was added to the tube of broken cells. Tubes were vortexed again for approximately 30 s and 350 µl chloroform was added, carefully vortexed to emulsify and microfuged for 10 min at maximum speed to separate the emulsion. The upper aqueous phase was carefully removed, placed into a new 1.5 ml Eppendorf microfuge tube and 500 µl isopropanol (−20 °C) were added to precipitate the DNA. Tubes were centrifuged for 5 min and supernatant was discarded. The DNA pellet was washed with 1000 µl of 70 % ethanol, centrifuged for 3 min and the supernatant was discarded. The DNA pellet was then dissolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). A 1:10 dilution of stock DNA in TE/10 buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) was the working stock of DNA. Methods for amplification of ITS and 28 S rDNA domains D1/D2 by PCR and sequencing with the ABI TaqDyeDeoxy Terminator Cycle sequencing kit/ABI Model 3100 or 3730 automated DNA sequencers were previously described (Peterson 2000).

PCR amplification

Concentrations of DNA and primers were determined and verified by agarose gel electrophoresis before doing PCR.

Symmetrical amplification was performed in a 96-well plate using primers described by Fedeshko (1992). Each PCR reaction mixture per well contained 4 μl of 10× PCR buffer, 1.6 μl $MgSO_4$ (50 mM), 0.8 μl deoxynucleotide (dNTP) mix (1.25 mM), 2.4 μl external 5′ end primer (IDH2195F) (5′-CAATGTGTCGTA CTGTGCCC-3′) (10 pmol μl^{-1}), 2.4 μl external 3′ end primer (IDH2793R) (5′-ACCTTCAGTCGCTGTTCCTC-3′) (10 pmol μl^{-1}), 0.32 μl Platinum Taq Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and 28.5 μl genomic DNA (ca. 50 ng) in TE/10 buffer. The plate was covered with a wax film, vortexed, and spun briefly in a centrifuge to collect the mixture in the bottom of the wells. The plate was put into a PCT-100™ Programmable Thermal Controller (MJ Research, Waltham, MA) and run using the following program: 35 × (94 °C, 1 min; 52 °C, 55 s; 72 °C, 2 min); 94 °C, 1 min; 52 °C, 55 s; 72 °C, 10 min. After amplification, all PCR products were visualized using agarose gel electrophoresis.

Agarose gel purification

If multiple bands of varying molecular weight were observed from the PCR products, gel purification of each band was required to determine if the products corresponded to the sequences of the *idh* gene. Gel purifications were done using an UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA), following the protocol included with the product. Briefly, PCR products were first separated in an agarose gel and using a UV light box, bands were cut from the gel and placed into separate 1.5 ml tubes. The UltraClean 15 Kit uses the silica binding particle method to purify DNA from agarose gels. Ultra SALT was added to the tubes which were heated to melt the agarose. The DNA was bound to UltraBIND silica particles in the presence of UltraSALT. The DNA/silica complex was pelleted by centrifugation and washed with UltraWASH. DNA was eluted from the silica by addition of water, followed by incubation and centrifugation; the supernatant containing the DNA was removed to a new tube.

Nucleotide sequencing

Sequencing of both strands of PCR products was performed using an Applied Biosystems MicroAmp 96-well plate and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). To each well was added: 3 μl distilled water (dH_2O), 2 μl BigDye, 2 μl Primer (1 pmol μl^{-1}) and 3 μl DNA (ca. 10 ng). The plate was covered with a wax film, vortexed and spun. The thermal cycler program had 24 cycles, consisting of a denaturation step at 94 °C for 30 s, an annealing step at 50 °C for 15 s, and an elongation step at 60 °C for 4 min. The cycling reaction product was purified and prepared for sequencing. To each well was added: 34 μl of 100 % ethanol and 4 μl of 10 M ammonium acetate; contents of the wells were mixed with a pipette tip. The plate was covered with a rubber mat and placed on a shaker for 20 min. The plate was centrifuged at 3600 rev min^{-1} for 45 min. The rubber mat was removed and the plate was inverted on a paper towel; the plate was then pulse spun. To each well was added 150 μl of 75 % ethanol, the rubber mat was replaced and the plate was centrifuged for 10 min. After centrifuging, the plate was allowed to drain over a paper towel and then pulse spun. The plate was dried in a swinging bucket vacuum centrifuge for 20–30 min. Ten microlitres of formamide (Hi-Di™, Applied Biosystems) were added to each well for sequencing. Samples were sequenced on an ABI 3730 DNA Analyser or ABI 3100 DNA Analyser. Sequence analysis was conducted using Sequencher software v4.2 (Gene Codes Corporation).

Isolation of genomic DNA for GeneWalking

Byssochlamys nivea ex-type strain NRRL 32565^T was inoculated into 50 ml malt extract (ME) broth and grown for 48 h at 25 °C shaken at 130 rev min^{-1}. A DNeasy Plant Mini kit (Qiagen, Valencia, CA) was used for extraction of DNA, according to manufacturer's specifications. Briefly, mycelia were filtered, frozen in liquid nitrogen, processed with AP1 buffer and RNase A and incubated at 65 °C to lyse the cells. AP2 buffer was added to the lysate mixture, mixed and put on ice for 5 min. The lysate was applied to a QIAshredder Mini Spin Column placed in a 2 ml collection tube and centrifuged. The flow-through was transferred to a new tube and Buffer AP3/E was added and mixed by pipetting. The mixture was applied to a DNeasy Mini Spin Column, sitting in a 2 ml collection tube, and centrifuged. The flow-through was discarded and the prior step was repeated. The DNeasy Mini Spin Column was placed in a new 2 ml collection tube, Buffer AW was added; after centrifuging, the flow-through was discarded. This step was repeated under slightly different conditions (centrifuging for 2 min at 20,000 × *g*, as opposed to 1 min at 6000 × *g*, previously) in order to dry the membrane. The DNeasy Mini Spin Column was transferred to a microfuge tube, Buffer AE was pipetted directly onto the DNeasy membrane, which was incubated at room temperature and centrifuged to elute; this step was repeated once. The DNA stock was stored at 4 °C to prevent shearing of DNA during freeze/thaw cycles.

Digestion of genomic DNA for GeneWalking

BD GenomeWalker™ Universal Kit from Clontech Laboratories (Mountain View, CA) was used to determine the remainder of the *idh* sequence, performing two walks. GenomeWalker libraries were constructed, according to manufacturer's specifications. Briefly, five separate reactions were set up for construction of four GenomeWalker libraries. One for each blunt-end restriction enzyme provided (*Dra* I, *EcoR* V, *Pvu* II, *Stu* I) and one for *Pvu* II digestion of human genomic DNA as a positive control. The reaction mixtures contained genomic DNA, restriction enzyme, restriction enzyme buffer and dH_2O. Reactions were gently mixed, incubated at 37 °C for 2 h, briefly vortexed at slow speed and stored at 37 °C overnight. To determine whether digestion was complete, 5 μl of each reaction product was run on a 1 % agarose gel.

Purification of DNA

To each reaction tube, 95 μl phenol was added, tubes were vortexed at slow speed and spun briefly to separate the aqueous and organic phases. The upper aqueous layer was transferred to a fresh 1.5 ml tube, chloroform was added, vortexed at a slow speed briefly and spun briefly to separate the aqueous and organic phases. The upper aqueous phase was removed

and put into a new tube, and ice-cold 95 % ethanol, 3 M NaOAc (pH 5) and glycogen were added. After vortexing and centrifuging, the supernatant was discarded and the pellet was washed with ice-cold 80 % ethanol and spun. After discarding the supernatant, the pellet was allowed to air dry. Once the pellet was completely dry, TE was added to dissolve the pellet and the tubes were vortexed.

Ligation of genomic DNA to BD GenomeWalker adaptors

Five ligation reactions were set up: four blunt-end digestions of the experimental genomic DNA and one positive control of the *Pvu* II digestion of human genomic DNA. Each tube contained digested, purified DNA, GenomeWalker Adaptor, 10× ligation buffer and T4 DNA ligase. The reactions were incubated overnight at 16 °C. To stop the reactions, the tubes were heated to 70 °C for 5 min, TE was added to each tube and the tubes were vortexed. GenomeWalker libraries were stored at −20 °C.

Primer design

Two gene specific primers were designed for each walk: one for primary PCR (GSP1) and one for secondary PCR (GSP2). Primers were designed based on the known sequence of *B. nivea* NRRL 32565^T. The nested PCR primer should anneal to sequences beyond the 3′ end of the primary PCR primer. Gene specific primers were 26–30 nucleotides in length and had a GC content of 40–60 %. Sequences at the 3′ end of gene-specific primers should not be able to anneal to the 3′ end of adapter primers. Primers used for Gene Walk 1 upstream were: BnGSP1-u (5′-GAACTCGCGACCCATGAAAGAACTCTG-3′) and BnGSP2-u (5′-GAAGCTGTCGTTCCAGTGCTCTGTCTC-3′). Primers used for Gene Walk 1 downstream were: BnGSP1-d (5′-TCCAGGA GTTGTCGCACTATTTAGGTC-3′) and BnGSP2-d (5′-TGAAGCT CATGCCGGAATGGGTAGAG-3′). Primers used for Gene Walk 2 upstream were: Bn2-GSP1-u (5′-CTTCTTTGGAGGCAACATTG AATGCAG-3′) and Bn2-GSP2-u (5′-CAGTAGGACACATTGGCT TCTTCCTG-3′). It was not necessary to perform Gene Walk 2 downstream to obtain the *idh* sequence.

PCR-based DNA walking in GenomeWalker libraries

The BD GenomeWalker DNA walking protocol consists of eight primary and secondary PCR amplifications: four experimental libraries, two positive controls, and two negative controls. The positive control gene-specific primers, PCP1 and PCP2, are provided. One microlitre of each library was used for the primary PCR; for secondary PCR, a 50× dilution of the primary PCR product was used. The reaction mixture per well contained: dH_2O, 10× BD Advantage 2 PCR Buffer, dNTP (10 mM each), Adaptor Primer 1 (10 μM), Advantage 2 Polymerase Mix (50×; Clontech Laboratories, Mountain View, CA), the appropriate gene-specific primer 1 and the DNA library, except for the negative controls which contained dH_2O. The wells were covered with a wax film and quickly vortexed and spun down. The plate was put in a thermal cycler using the following program: 7 × (94 °C, 25 s; 72 °C, 3 min), 32 × (94 °C, 25 s; 67 °C, 3 min), and 67 °C for an additional 7 min after the final cycle. Products were visualized by agarose gel electrophoresis. If no products were visible, the plate was placed back in the thermal cycler for an additional five cycles. If there still were not any products, new reactions were repeated with the following program: 7 × (94 °C, 25 s; 70 °C, 3 min), 32 × (94 °C, 25 s; 65 °C, 3 min), and 65 °C for an additional 7 min.

Products from the primary PCR were diluted 50× and were used in the secondary PCR reactions. Each reaction well contained dH_2O, 10× Advantage 2 PCR buffer, dNTP (10 mM each), Adaptor Primer 2 (10 μM), Advantage 2 Polymerase Mix (50×), appropriate gene specific primer 2, and diluted primary PCR product. The wells were covered with a wax film, vortexed and spun down. The thermal cycler program was as follows: 5 × (94 °C, 25 s; 72 °C, 3 min), 20 × (94 °C, 25 s; 67 °C, 3 min) and an additional 7 min at 67 °C. Products were visualized with agarose gel electrophoresis. If no products were observed, the plate was placed back in the thermal cycler for an additional five cycles. If still no product was visible, the secondary PCR would be repeated with the program: 5 × (94 °C, 25 s; 70 °C, 3 min), 20 × (94 °C, 25 s; 65 °C, 3 min), with an additional 7 min at 65 °C. Products were run on a gel and each band was cut out and gel purified. Purified products were cycle sequenced, as described previously. The complete protocol for the GeneWalking kit is available at www.clontech.com/clontech/techinfo/manuals/PDF/PT1116-1.pdf.

Based on the sequence obtained from GeneWalking of *B. nivea* NRRL 32565^T, primers were designed to determine by PCR the complete *idh* sequences for the remaining *B. nivea* strains and to verify the sequence that had been obtained by Gene Walking. The primers used were the following: BnIDH1F (5′-CAGACACCTTAATAATCTAACTC-3′) and BnIDH629R (5′-CATGAAAGAACTCTGCACGATG-3′); BnIDH18F (5′-CTCATC AAGATGGTTCTGG-3′) and BnIDH912R (5′-AGCTGGCCAGTG GGCTTGCTAG-3′); BnIDH26F (5′-GATGGTTCTGGAAACAG GACTG-3′) and BnIDH616R (5′-TGCACGATGATCGATGCTTT GAG-3′); BnIDH141F (5′-AGGCGATGGTGCACCAATTCC-3′) and BnIDH912R (5′-AGCTGGCCAGTGGGCTTGCTAG-3′); BnIDH170F (5′-GAAGCCAATGTGTCCTACTGC-3′) and BnIDH1003R (5′-GA AGCTAAGATTTGATCTCAC-3′); and BnIDH170F (5′- GAAGCC AATGTGTCCTACTGC-3′) and PgIDH2887R (5′-CAACGTGA ATTCCGCCATCAACCAAC). Numbers used to identify the primers refer to a specific position in the *B. nivea idh* sequence, with the exception that the numbers 1 through 26 represent bases 5′ of the ATG start codon of the *idh* gene. The sequence for *P. griseofulvum* NRRL 2159A (GenBank accession number AF006680) was used to design primer PgIDH2887R.

Patulin production

Spores (final concentration of 2×10^6 spores ml^{-1} growth medium) were harvested and inoculated in 250 ml Erlenmeyer flasks containing 50 ml potato dextrose broth (PDB) supplemented to contain 152 μM $MnCl_2 \cdot 4\,H_2O$. Cultures were grown in duplicate at 30 °C on a rotary shaker (240 rev min^{-1}) for a total of 14 d. To measure patulin production at 5, 10 and 14 d, 3–4 ml aliquots were aseptically removed from shake flasks, filtered and analysed. Fungi were also incubated on slants of MEA at 40 °C to determine ability to grow at high temperatures.

Patulin extraction

The protocol used for the extraction and detection of patulin was described by Dombrink-Kurtzman & Blackburn (2005) and based on methods developed by Trucksess & Tang (1999) and modified by Eisele & Gibson (2003). Briefly, filtered growth medium samples were extracted, using solid-phase extraction (SPE) columns (Waters Oasis HLB, Waters, Milford, MA) and a vacuum manifold box. Before use, the columns were conditioned successively with dH_2O, methanol, and dH_2O. Samples were loaded in the SPE columns, which were rinsed with 1 % sodium bicarbonate, followed by acetic acid and dried before eluting with 10 % ethyl acetate in ethyl ether. Vials were evaporated to dryness under nitrogen at room temperature and immediately suspended in 0.1 % acetic acid.

Determination of patulin

The patulin stock standard solution (200 $\mu g\,ml^{-1}$) was prepared using 5 mg pure crystalline patulin (Sigma Chemical Company, St Louis, MO), diluted with 25 ml ethyl acetate and stored at $-20\,^{\circ}$ C. The working standard contained 10 $\mu g\,ml^{-1}$. For hplc analyses, the calibration standards were diluted in 0.1 % acetic acid. Chromatographic analysis was performed as described previously by Dombrink-Kurtzman & Blackburn (2005), using a high-performance liquid chromatograph (SpectraSYSTEM/Thermo Finnigan, San Jose, CA), equipped with P2000 solvent delivery system, AS3000 autosampler and UV6000LP photodiode array detector monitoring at both 276 nm and acquiring UV–visible scanning (220–360 nm) data. A Zorbax StableBond-Aq (SB-Aq) column (Agilent Technologies, Wilmington, DE) and a guard cartridge were used; the mobile phase was dH_2O, acetonitrile and 50 $mmol\,l^{-1}$ potassium phosphate buffer, pH 2.3 (85:5:10), run at ambient temperature with a flow rate of 1.0 $ml\,min^{-1}$.

Results

DNA sequence comparisons

Six strains of *B. nivea* had been obtained from Culture Collections in the United States and Europe. Two additional strains identified as *B. nivea* and *B. fulva* were received from the University of California-Davis Herman Phaff Culture Collection and were included in the study; it was subsequently determined by sequencing of rDNA that both of these strains were *B. nivea*. DNA was isolated from the strains and the nucleotides in the ITS1, 5.8 S RNA, ITS2 regions and the partial sequence of the LSU (28 S) rDNA were sequenced and compared. In the aligned rDNA data set composed of a contiguous region from the 3′ terminal bases of the 18 S rDNA through ITS1, 5.8 S, ITS2 and the 5′-end of 28 S rDNA, there was only a single base difference in the entire rDNA region examined; *B. nivea* NRRL 32565^T and NRRL 35592 both had a G at position 271 in the 28 S rDNA sequence, whereas an A was present at that position in the other six strains. Analysis of the entire *idh* nucleotide sequence of all *B. nivea* strains revealed total identity for both the protein coding regions and the two introns.

Comparison was made of the amino acid sequence corresponding to the *idh* gene of *B. nivea*, *P. expansum* and *P. griseofulvum* (Table 2). Previous research had indicated that the *P. expansum* strains and the *P. griseofulvum* strains contained the same amino acid sequence at the species level (Dombrink-Kurtzman in press). A strain of *P. expansum* (GenBank accession number DQ084388) was included in the comparison. All of the nine conserved amino acids, described by Fedeshko (1992), were present in all of *P. griseofulvum* strains, but strains of *B. nivea* and *P. expansum* contained one amino acid difference (position 46 in the IDH amino acid sequence) where lysine was replaced by threonine. There was a difference of 15 amino acids in the *idh* sequences of *P. expansum* NRRL 35231 and *P. expansum* (DQ084388).

The first successful *B. nivea idh* sequences were obtained for strains NRRL 35593 and NRRL 35233, using primers IDH 2195F and IDH 2793R described by Fedeshko (1992). From the sequences obtained, primer IDH2444F was designed (5′-ATGCACATGGAAGGCGAGAC-3′) and used with primer IDH2887R (5′-CAACGTGAATTCCGCCATCAACCAAC-3′). The latter primer was designed based on the nucleotide sequence of *P. griseofulvum* (GenBank accession AF006680) and was able to work well with strains of *B. nivea*. A segment of approximately 500 bp in length was obtained for seven of the *B. nivea* strains and resulted in determination of the 3′ sequence of the *idh* gene. GeneWalking was then used to determine the remaining 5′ and 3′ sequences of the *idh* coding region of *B. nivea* NRRL 32565^T. Gene Walk 1 and Gene Walk 2 were done for the area 5′ upstream; only Gene Walk 1 was done for the area 3′ downstream to obtain the *idh* sequence. It was not necessary to perform Gene Walk 2 downstream to obtain the *idh* sequence. Primers designed based on the complete *idh* coding region were used to determine the *idh* gene sequences of the remaining seven strains of *B. nivea*. All strains contained identical sequences for both intron 1 and intron 2 (Table 3). Intron 2 was unusually long compared with intron1 of *B. nivea* and contained 139 nucleotides. Intron 1 was comparable in size to the number of nucleotides present in introns 1 and 2 of *P. expansum* and *P. griseofulvum* (Dombrink-Kurtzman 2006).

Patulin determination

Maximum concentrations of patulin produced by the four *B. nivea* strains examined were similar to those of strains of *P. expansum* (Dombrink-Kurtzman & Blackburn 2005). Minimal amounts of patulin were detected at 5 and 14 d following inoculation, whereas maximum patulin production occurred at 10 d (Fig. 1). The highest levels of patulin (367.73 and 211.54 $\mu g\,ml^{-1}$) were detected in *B. nivea* strains NRRL 32565^T and NRRL 2615, respectively. Two strains (accessioned as NRRL 35592 and NRRL 35593), obtained from the University of California-Davis Culture Collection as *B. nivea* (40–200) and *B. fulva* (55–85), respectively, also produced high levels of patulin (223.99 and 102.08 $\mu g\,ml^{-1}$). There appeared to be a pH correlation for patulin production because strains producing the highest levels of patulin had a final pH of 4 or 5, whereas all strains producing low levels of patulin, or none at all, had a higher pH (Dombrink-Kurtzman & Blackburn 2005). Patulin

Table 2 – Multiple sequence alignment of deduced amino acid sequences of the *idh* genes of *Byssochlamys nivea*, *Penicillium griseofulvum* and *P. expansum*

Bn 32565	MVLETGLKGA	HVLITGGTRG	MGQAMVHQFL	QEEANVSYCA	RTVTNTEFDE
Pg 2159A	...T......	....	..E....K..	E.........	.....K....
Pe 35231	...	....	..E....K..	....	.......Y.D
Pe GB	...I......	....	..E....K..	....	.......Y.D
Bn 32565	FHKMLPEGNR	ARAVGTAFNV	ASKEAIIDWV	KSSAERVGKI	DVIIANASPM
Pg 2159A	..AT.....T	.........D.	...D..VQ..	E.....L.R.	....
Pe 35231	.YST.A...T	.........D.	...DSLVK..	E.....L.R.	....
Pe GB	.YPT.AK..T	.........D.	..ID.LVK..	E.......R.	....
Bn 32565	HMEGETEHWN	DSFAIDVMGF	VELVAAATPY	LEKSPQASII	VQSSFMGREF
Pg 2159A	.........E	S.........	....R.....	....	....
Pe 35231	.........E	S.........	....K.....	....	....
Pe GB	.......D.E	N.........	...AR.....	....	....
Bn 32565	YRSPPAAYGP	CKAAQLQHVQ	ELSHYLGPKG	IRVNAISPGP	VLCEGGPWEK
Pg 2159A	....	....	....F.....	....	I..K.....L
Pe 35231	....	....	....F.....	....	...K.....L
Pe GB	....	....	....F.....	....	I..KN....L
Bn 32565	YLKLMPEWVE	EQRLKIPLKR	LGGPKEVANV	AVFLASPLAS	FVTGTNVLVD
Pg 2159A	.S.IN.....	.....V....	....T...A.	....	..S...M...
Pe 35231	.S.IN.....	....	....T.....	....	..S...M...
Pe GB	.S.TN.....	....	..C.T...A.	....	......M...
Bn 32565	GGIHVGTQF				
Pg 2159A	....				
Pe 35231					
Pe GB	...TF....				

Bn, *Byssochlamys nivea* GenBank DQ322212; *Pg*, *Penicillium griseofulvum* GenBank AY885567.1; *Pe*, *P. expansum* GenBank AY885569.1; *Pe* GB, *P. expansum* GenBank DQ084388. The nine amino acids shown in bold have been described by Fedeshko (1992) as being conserved amino acids. Arginine and lysine are underlined because they represent basic residues that are present in NADP-specific dehydrogenases. In the *idh* genes of both *B. nivea* and *P. expansum*, the conserved lysine residue has been replaced by threonine. It has been proposed that the basic residues are involved in the stabilization of the negative charge on the NADP molecule (Scrutton *et al.* 1990).

Table 3 – Introns 1 and 2 of *Byssochlamys nivea* strains

B. nivea NRRL 32565^T	Intron 1
GTATGATGCTCATTTTCTTACCTTACGTCACTGACTAC ATTCACCAG	
B. nivea NRRL 32565^T	Intron 2
GTAACCCCCCTCTTTCTCTCCTCTGCCTTCCCATTATT CCAATTATAATCTTTTATCTCTAATTCCTCTGGCTCCC CTCGCCTACGTATTTTATTCCCCACGACTTTCGAGAAC TTCCCTCCTGACAGAAGATATACAG	

is stable at acidic pH, but at alkaline pH, it is unstable. All *Byssochlamys nivea* strains were able to grow at 30 °C; *B. nivea* NRRL 32565^T was able to grow at 40 °C.

Discussion

Analysis of the entire *idh* nucleotide sequence of all *B. nivea* strains revealed total identity for both the protein-coding regions and the two introns. Prior studies with strains of *P. griseofulvum* and *P. expansum* (Dombrink-Kurtzman 2006) had shown identical amino acid composition at the species level, but differences that existed at the nucleotide level did not affect the coding of the amino acids. All three of the *P. expansum* strains were identical to each other for both introns 1 and 2, but there were nine additional nucleotides in intron 1, compared with the number of nucleotides in intron 1 of the *P. griseofulvum* strains (Dombrink-Kurtzman 2006). For the *B. nivea* strains examined in this study, intron 2 was nearly three-fold greater in size (139 nucleotides) than intron 2 in strains of *P. griseofulvum* and *P. expansum*. By constructing GeneWalker libraries and using them to produce products that were then sequenced, the coding-regions of the *idh* genes for all eight strains of *B. nivea* were obtained. This allowed the comparison of *idh* sequences from three different species, *B. nivea*, *P. expansum* and *P. griseofulvum*.

It was noted that the *idh* nucleotide sequence of *B. nivea* resembled that of *P. expansum* at a conserved position for an amino acid (position 46). The conserved basic amino acid (lysine) is found only in $NADP^+$ specific dehydrogenases, but it has been substituted by threonine in strains of *B. nivea* examined here, as well in strains of *P. expansum* (Dombrink-Kurtzman 2006). The conserved basic amino acids, arginine and lysine, are thought to be involved in stabilizing the extra negative phosphate charge on the NADP molecule in dehydrogenases requiring NADP as a reducing coenzyme (Scrutton *et al.* 1990). It may be that the amount of patulin produced is influenced by the presence of the two conserved basic amino acids (arginine and lysine). *P. griseofulvum* strains had both conserved basic amino acids present; the amount of patulin produced by strains of *P. griseofulvum* was greater than the amount produced by strains of *P. expansum* (Dombrink-Kurtzman & Blackburn 2005).

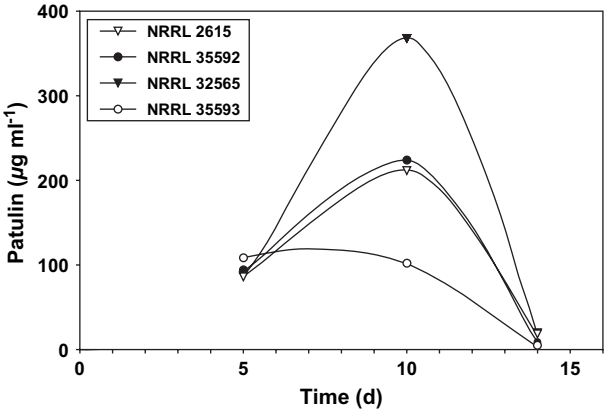

Fig 1 – Patulin production by *B. nivea* NRRL 32565^T (▼), *B. nivea* NRRL 2615 (△), *B. nivea* NRRL 35592 (●) and *B. nivea* NRRL 35593 (○).

P. expansum is frequently mentioned as being the fungus most commonly responsible for patulin production in food products, mainly apple juice and apple products. The results of this study indicate that strains of *B. nivea* may be more important agents responsible for the presence of patulin because their ascospores can survive the temperatures used for pasteurization (Tournas 1994). *Byssochlamys* species are able to grow at a pH as low as 2.0 and are saprophytic, usually contaminating fruit on the ground. Heat-resistant fungi are widely distributed in soil and can result in spoilage problems in products containing fruits that have been contaminated by soil. Ripe apples used to produce apple juice concentrates are the most commonly affected fruit. Both *B. nivea* and *B. fulva* are capable of forming heat-resistant ascospores and are commonly responsible for spoilage of canned fruit and fruit products. It is not advisable to destroy *Byssochlamys* ascospores in fruit products by heat alone because the sensory quality of the products will be affected by the time and temperature required. Outgrowth of heat-resistant ascospores after thermal processing occurs with the production of mycelium and the subsequent production of patulin. Post-harvest preventative measures regarding apples in storage include the increasing use of controlled atmospheres (CA) to supplement good refrigerated storage practices. In CA controlled storage, a beneficial combination of reduced oxygen levels and increased carbon dioxide concentrations in the atmosphere are being combined with refrigeration.

This research was undertaken to identify a specific gene (*idh*) present in the biosynthetic pathway of fungi capable of synthesizing the mycotoxin patulin. By identifying a particular nucleic acid sequence common to all fungi capable of producing patulin, but not present in other fungi, it should be possible to design a DNA-based probe to test for the presence of these fungi in fruit products, specifically apple juice. There have been no reports of the sequence of the *idh* gene in *B. fulva*, but it has been associated with patulin production in fruit juices (Rice *et al.* 1977). Determination of the *idh* sequence of *B. fulva* is planned. All of the *B. nivea* strains tested contained the *idh* gene, which had 88 % homology to strains of *P. expansum* and *P. griseofulvum* previously examined (Dombrink-Kurtzman 2006). It is remarkable that the *idh* sequence for all eight of the strains of *B. nivea* were identical, even though they had been isolated worldwide. Because patulin is one of two mycotoxins presently regulated by the US Food and Drug Administration, means to identify and control its presence in food for humans would be beneficial.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study for the *idh* gene have been deposited with GenBank and have been assigned the following GenBank accession numbers: *Byssochlamys nivea* NRRL 32565^T (DQ322212), *B. nivea* NRRL 2615 (DQ322209), *B. nivea* NRRL 29820 (DQ322210), *B. nivea* NRRL 32294 (DQ322211), *B. nivea* NRRL 35216 (DQ322213), *B. nivea* NRRL 35233 (DQ322214), *B. nivea* NRRL 35592 (DQ322207) and *B. nivea* NRRL 35593 (DQ322208).

The nucleotide sequences determined in this study for the ITS/partial LSU (28 S) rDNA genes have been deposited with GenBank and have been assigned the following GenBank accession numbers: *Byssochlamys nivea* NRRL 32565^T (DQ322219), *B. nivea* NRRL 2615 (DQ322222), *B. nivea* NRRL 29820 (DQ322218), *B. nivea* NRRL 32294 (DQ322216), *B. nivea* NRRL 35216 (DQ322221), *B. nivea* NRRL 35233 (DQ322217), *B. nivea* NRRL 35592 (DQ322220) and *B. nivea* NRRL 35593 (DQ322215).

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